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Eukaryotic Fatty Acylation Drives Plasma Membrane Targeting and Enhances Function of Several Type III Effector Proteins from *Pseudomonas syringae*

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Summary

Bacterial pathogens of plants and animals utilize conserved type III delivery systems to traffic effector proteins into host cells. Plant innate immune systems evolved disease resistance (*R*) genes to recognize some type III effectors, termed avirulence (Avr) proteins. On disease-susceptible (*r*) plants, Avr proteins can contribute to pathogen virulence. We demonstrate that several type III effectors from *Pseudomonas syringae* are targeted to the host plasma membrane and that efficient membrane association enhances function. Efficient localization of three Avr proteins requires consensus myristoylation sites, and Avr proteins can be myristoylated inside the host cell. These prokaryotic type III effectors thus utilize a eukaryote-specific posttranslational modification to access the subcellular compartment where they function.

Introduction

Plants can specifically resist infection by bacterial pathogens through the interaction of host resistance (*R*) genes and pathogen avirulence (*avr*) genes. The simplest mechanistic interpretation of these genetic systems is that Avr and R proteins interact directly, although this has been difficult to generalize experimentally (see Scofield et al., 1996; Tang et al., 1996 for the exception). Interaction of Avr and R proteins, potentially in a multiprotein complex, results in disease resistance responses characterized by a suite of biochemical events often culminating in both host cell death (hypersensitive response, HR) at the site of infection and cessation of pathogen growth (Yang et al., 1997; Scheel, 1998). If alternate alleles of either the *R* or *avr* genes are expressed during this interaction, then there is no recognition and successful infection of the plant by the bacteria ensues.

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It is puzzling that phytopathogens express Avr proteins which can condition the pathogen's demise. Some *avr* genes contribute to successful infections on susceptible hosts, ensuring a continued advantage for bacteria containing them (Kearney and Staskawicz, 1990; Lorang et al., 1994). For example, *avrRpm1* from *Pseudomonas syringae* pv. *maculicola* strain M2 (PsmM2) is a virulence factor. PsmM2 requires *avrRpm1* to grow optimally on *Arabidopsis* plants lacking the corresponding *RPM1 R* gene (Ritter and Dangl, 1995). It is generally true that a given *avr* gene is not widely dispersed among isolates of Pseudomonads. This suggests that a battery of genes, dispersed among pathogen isolates, contributes quantitatively to the virulence of any given strain. For example, *avrRpm1* is present in only 5 of 20 *P. syringae* pv. *maculicola* strains analyzed (Dangl et al., 1992), yet strains that lack it are still pathogenic.

Bacterial *avr* genes are part of the *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity) regulon (Huynh et al., 1989; reviewed by Alfano and Collmer, 1997). In *P. syringae*, this regulon encodes linked transcriptional regulators and the structural proteins for an evolutionarily conserved type III secretion apparatus. Bacterial virulence factors that modulate or usurp host mammalian cell functions are trafficked to the interior of host cells via the type III pilus. These type III effectors have targets inside eukaryotic host cells (see Cornelis and Wolf-Watz, 1997; Galan and Collmer, 1999 for reviews). Both Avr proteins and known type III effectors from animal pathogens can be secreted from phytopathogenic bacterial cells in a type III–dependent manner (Anderson et al., 1999; Rossier et al., 1999). Thus, Avr proteins are type III effector proteins.

Avr-R recognition can occur inside the plant cell. Following expression of a bacterial *avr* gene using plant transcriptional control signals, Avr proteins can elicit an HR-like cell death in plant cells expressing the appropriate *R* gene (reviewed in Mudgett and Staskawicz, 1998). Curiously, expression of Avr proteins in disease-susceptible plants can lead to delayed, weak cytotoxic effects, suggesting that Avr proteins may have additional targets inside the plant cell (Gopalan et al., 1996; McNellis et al., 1998). Based on analogy to mammalian pathosystems, we and others infer that type III effectors from phytopathogens are translocated into the host cell, although direct demonstrations of this are lacking. For example, a cleaved form of the *P. syringae* AvrRpt2 protein is detected inside plant cells and not in bacterial lysates following type III–dependent delivery (Mudgett and Staskawicz, 1999). This result argues strongly for delivery of AvrRpt2 into the plant cell before, or concomitant with, its proteolytic cleavage. Despite these recent advances, little is known about the subcellular localization, and hence site of action, of the phytopathogen type III effector proteins inside the plant cell. The related *Xanthomonas* proteins AvrBs3 and PthA have nuclear localization sequences that are required for both avirulence function when delivered in a type III–dependent manner, and for nuclear localization following expression inside the plant cell (Yang and Gabriel, 1995; Van den

Table 1. *P. syringae* Avirulence Genes Contain N-Terminal Consensus Eukaryotic Fatty Acylation Sequences

	1 2 3 4 5 6 7 8 9
avrRpm1	M G C V S S T S R
avrB	M G C V S S K S T
avrPphB[a]	G C A S S G V S
SOS3 Ca sensor	M G C S V S K K K
avrC	M G N V C F R P S
avrPto	M G N I C V G G S
CPK1	M G N T C V G P S

References for each sequence are (top to bottom): Dangl et al., 1992; Tamaki et al., 1988; Jenner et al., 1991; J.-K. Zhu, personal communication; Tamaki et al., 1988; Salmeron and Staskawicz, 1993; Ellard-Ivey et al., 1999.

[a] Represents N terminus of processed AvrPphB protein as determined by Puri et al., 1997.

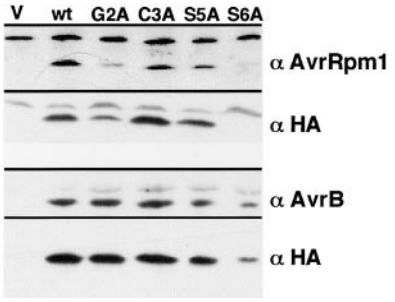

Figure 1. Expression of Native and HA Epitope–Tagged AvrRpm1 and AvrB in *P. syringae* DC3000

Extracts were prepared from Pst DC3000 expressing empty vector (V) or the wild-type (wt) and mutant Avr proteins indicated at top. Duplicate blots were probed with either native antisera to AvrRpm1 or AvrB or the monoclonal anti-HA antibody, as listed on the right. Lanes were equally loaded. Top two blots: AvrRpm1, bottom two blots: AvrB.

Ackerveken et al., 1996). Presumably, these Avr proteins interact with host nuclear factors, potentially influencing host defense gene transcription (Zhu et al., 1999).

We noted a subset of *P. syringae* type III effectors (Table 1) with predicted N-terminal eukaryotic consensus sequences for fatty acylation, modifications that promote plasma membrane association. This subset includes both AvrRpm1 and AvrB from *P. syringae* pv. *maculicola* and *P. syringae* pv. *glycinea*, respectively, which are recognized in *Arabidopsis* by *RPM1* (Bisgrove et al., 1994; Grant et al., 1995). AvrRpm1 and AvrB share no homology other than this N-terminal sequence, including the G2 residue known to be the target for covalent myristoylation (a $C_{14:0}$ acyl group; Johnson et al., 1994). An apparent exception to the G2 rule is the AvrPphB protein from *P. syringae* pv. *phaseolicola*. AvrPphB expresses a glycine not at its translational N terminus, but rather at the N terminus of an intramolecular cleavage product (Puri et al., 1997). Myristoylation often occurs in conjunction with palmitoylation, a $C_{16:0}$ lipid attachment at C3 or C5 (Resh, 1994), and the proteins in Table 1 feature this residue. Both fatty acylation events are specific to eukaryotes, notably Gα subunits and Src family tyrosine kinases (Johnson et al., 1994; Resh, 1994). *Arabidopsis* proteins in Table 1 use these N-terminal sequences as acylation sites (Ellard-Ivey et al., 1999; J. K. Zhu, personal communication). Thus, plant consensus acylation sites are typical of those from other eukaryotes. We addressed whether or not these amino acid residues are important for Avr protein function, and whether these pathogen effectors are targeted to the host plasma membrane. RPM1 is enriched in plasma membrane vesicles from plant cells (Boyes et al., 1998), making it likely that the Avr proteins it recognizes would also localize there.

Results

Consensus Eukaryotic Fatty Acylation Sites Mediate Type III–Dependent Delivery of AvrRpm1 and AvrB Function

We introduced site-specific alanine exchanges G2A, C3A, S5A, and S6A into both AvrRpm1 and AvrB and expressed these from the native *avrRpm1* promoter, with or without a C-terminal HA epitope tag (Experimental Procedures). We assessed whether these exchanges affected protein production in *P. syringae* pv. *tomato* (Pst) DC3000. Figure 1 demonstrates that either antisera to each Avr protein, or the anti-HA epitope monoclonal antibody, recognized proteins of the correct apparent molecular weight (AvrRpm1 at 29 kDa, AvrB at 36 kDa) that are not present in bacterial extracts made from cells carrying an empty vector. The G2A mutant of AvrRpm1 accumulated to variably lower levels than wild type, but we did not observe similar decreases for the AvrB G2A mutant protein. The C3A exchanges in either gene were as stable as wild type. Surprisingly, S6A exchange in either AvrRpm1 or AvrB significantly reduced protein accumulation, and interpretation of subsequent functional data must bear this in mind. Structural signals comprising at least 15 codons mediate type III–dependent secretion or delivery of effector proteins into host cells (Anderson et al., 1999). We expressed wild-type and mutant proteins in an *E. coli* strain expressing either a wild-type or mutant *Erwinia chrysanthemi* type III secretion system previously used to monitor AvrB secretion (Ham et al., 1998). We monitored *hrp*-dependent AvrB secretion and found that wild-type and mutant proteins were secreted equally in an *hrp*-dependent manner (data not shown). However, we were unable to observe secretion of wild-type AvrRpm1, consistent with the observation that different type III effectors are secreted with different efficiencies (e.g., Ham et al., 1998).

We tested delivery of AvrRpm1 or AvrB avirulence function to an *Arabidopsis* accession (inbred line), Col-0, which expresses *RPM1*. We monitored in planta growth of strains expressing the various avirulence protein derivatives (Figure 2A, top). Virulent Pst DC3000 carrying an empty vector grow ~1000-fold over three days. Expression of wild-type *avrRpm1* or *avrB* in Pst DC3000 decreases pathogen growth by ~100 fold. This reflects recognition of AvrRpm1 or AvrB via *RPM1*. In contrast, the G2A derivative of either AvrRpm1 or AvrB is not recognized efficiently by the host, and pathogen growth is unhindered. The S6A mutation eliminated avirulence, but interpretation of this data is compromised by the diminished levels of S6A accumulation noted above. We also monitored the onset of HR following

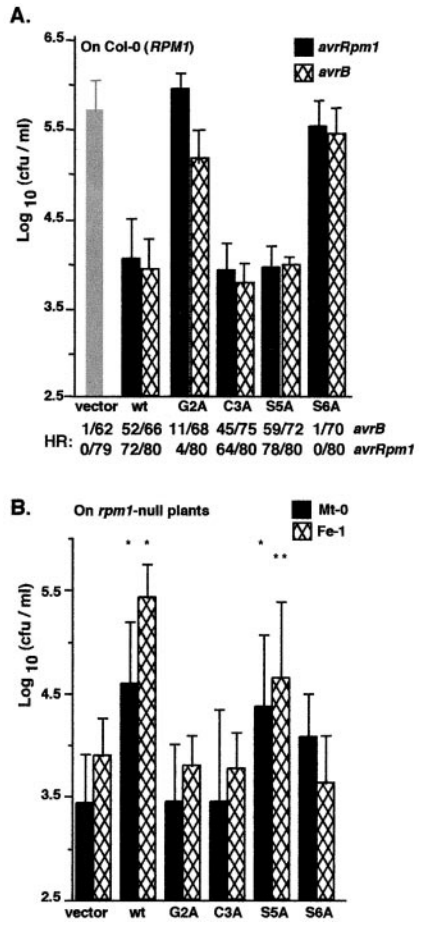

Figure 2. Maximal Type III–Dependent AvrRpm1 and AvrB Effector Functions Are Mediated by Consensus Acylation Sites

(A) The G2A and C3A exchanges reduce the avirulence functions of AvrRpm1 and AvrB. Col-0 (*RPM1*) leaves were inoculated with Pst DC3000 carrying either empty vector (V), the wild-type (wt) or mutant *avr* derivatives as listed on the x axis (initial inoculum of $\sim 1 \times 10^5$ cfu/ml). Bacterial titers three days post inoculation (dpi) are graphed on the y axis. Mean and standard deviation from 3 independent experiments. Day 0 titers ranged from $\log_{10} = 2.5$–3.2. The number of HR^+ leaves/total inoculated leaves scored at 5 hr post inoculation (hpi) using high initial inoculum ($OD_{600} = 0.05$ for AvrB and $OD_{600} = 0.1$ for AvrRpm1) is displayed below the corresponding growth data. Data are summed from three different experiments. (B) The G2A and C3A exchanges reduce the virulence function of AvrRpm1. In planta growth assay on *rpm1* null accessions Mt-0 and Fe-1 plants as in (A) but with an initial inoculum Psm CR299 of 1.0×10^3 cfu/ml. Mean and standard deviation from four independent experiments at 3 dpi Student's t test for significance of differences to vector control was $p < 0.1$ (*) and $p < 0.15$ (**).

inoculation of a high dose of *P. syringae* expressing the various avirulence proteins. Expression of either wild-type *avrRpm1* or *avrB* in *Pst* DC3000 triggers HR on Col-0 at 5 hr post inoculation, while empty vector did not (Figure 2A, bottom). G2A exchange in either AvrRpm1 or AvrB significantly lowers the percentage of leaves responding. While the effects of G2A exchanges are not complete in this assay, they are at the lower titers used for in planta growth. Additionally, C3A exchange in AvrB and AvrRpm1 reproducibly resulted in both fewer responding leaves, and a slightly delayed response. We conclude from Figure 2A that the consensus myristoylation sites of both AvrRpm1 and AvrB are required for full avirulence function following type III–mediated delivery to *Arabidopsis*.

Several *P. syringae* type III effector proteins, including AvrRpm1, serve as virulence factors during infection of plant genotypes lacking the appropriate *R* gene product. Loss of function Col-0 *rpm1* mutant alleles exist, and several *Arabidopsis* accessions have a naturally occurring deletion allele (*rpm1* null; Grant et al., 1995). PsmM2 is pathogenic on *rpm1* null *Arabidopsis* accessions like Mt-0, Fe-1, and Cvi-0. A Tn3*spice* insertion into *avrRpm1* in Psm M2 (giving rise to strain CR299) decreased virulence in a dose-dependent manner (Ritter and Dangl, 1995). The essence of this finding is displayed in Figure 2B, left, where expression of wild-type *avrRpm1* rescues CR299 to full virulence on *rpm1* nulls Mt-0 and Fe-1. Figure 2B demonstrates that both G2A and C3A exchanges significantly reduce the virulence function of wild-type *avrRpm1* as measured by CR299 growth. We conclude from this experiment that both myristoylation and palmitoylation consensus sites are important for maximal AvrRpm1 virulence function when delivered via the type III system to *Arabidopsis*. Similar experiments with AvrB were not performed, as no obvious virulence activity has been ascribed to AvrB on *Arabidopsis*.

Consensus AvrRpm1 and AvrB Acylation Sites Are Required for Maximal *RPM1* Function by Following *avr* Expression Inside Host Cells

Our genetic experiments suggested that acylation of the AvrRpm1 and AvrB type III effector proteins might mediate their function. Unfortunately, it has proven impossible to directly detect type III–dependent delivery of effector proteins from plant pathogenic bacteria to plant host cells. However, expression of putative type III effector proteins like AvrRpm1 an AvrB inside *RPM1* accessions like Col-0 results in an HR-like response (Gopalan et al., 1996; Leister et al., 1996). We used *Agrobacterium* to deliver the *avr* genes and a dexamethasone (DEX)-inducible vector system to conditionally express them from the transferred T-DNA (Aoyama and Chua, 1997; Experimental Procedures). If mutant derivatives of AvrRpm1 and AvrB unable to trigger *RPM1*-dependent resistance when delivered from *P. syringae* also proved unable to initiate an *RPM1*-dependent response when delivered from *Agrobacterium*, then two conclusions could be drawn: first, that the mutant phenotypes were unlikely to be a consequence of altered delivery via the *P. syringae* type III system and second, that localization of Avr proteins delivered via *Agrobacterium* should reflect the natural localization during *P. syringae* infection.

Figure 3A demonstrates that DEX-induced transient expression of either AvrRpm1 or AvrB initiates an *RPM1*-dependent response. We used two sets of isogenic plants for this experiment: first, wild-type Col-0 (*RPM1*) and an isogenic loss-of-function *rpm1*-fs allele, and second, the *rpm1* null accession Fe-1 and a transgenic Fe-1 expressing *RPM1*. Figure 3B illustrates that G2A exchange in either Avr protein significantly reduced the ability to trigger an *RPM1*-dependent response. Avr protein levels in these leaves, however, were undetect-

able (not shown). Our inability to detect Avr protein in this assay suggests that low Avr protein levels are sufficient to trigger *RPM1*-dependent cell death. DEX treatment does not induce Avr protein accumulation in cultured *Agrobacterium* (see Experimental Procedures).

The Consensus Myristoylation Site Mediates Maximal *rpm1*-Independent Cytotoxicity Triggered by AvrB Expression Inside Host Cells

We assayed a series of *rpm1* loss-of-function or null alleles using the same expression system. We reasoned that Avr proteins might be sufficient to trigger a cellular response indicative of their virulence function, based on the slow cytotoxicity indicative of type III effector action in susceptible animal cells (see Introduction). Figure 4A demonstrates that AvrB is sufficient to trigger chlorosis mediated by a slow cytotoxic response in the absence of *RPM1* (measured by Trypan blue uptake, not shown). Surprisingly, these responses are polymorphic with respect to host genotype. Thus, AvrB expression initiates a chlorotic response on all accessions tested (including Nd-0; see Gopalan et al., 1996) except the *rpm1* null Cvi-0. AvrRpm1 can sometimes trigger a response in the *rpm1* null accessions Mt-0 and Aa-0, but this phenotype has proven unreliable and will not be discussed further. The fact that AvrRpm1 and AvrB protein both accumulated in Cvi-0 (see below) argues against general cytotoxicity as the cause of the *rpm1*-independent response. Response to AvrB in a cross between the Col-0 *rpm1*-fs allele and Cvi-0 indicates either one or two host genes segregating which control this trait (F1: 8/8 responding; F2: 154 responding, 36 not responding, χ^2 for 3:1 = 3.42, p = 0.05; χ^2 for 13:3 = 0.0, p = 0). We mapped a locus near the chromosome 5 marker *SPL2* which controls this response (linkage = 14.7 map units; Z. N. and J. L. D., unpublished). Note that *RPM1* maps to chromosome 3, proving that this response is not due to residual RPM1 activity in the *rpm1*-fs allele. While we cannot exclude a weak *R* gene effect as an explanation of this phenotype, preliminary transcriptional profiling suggests that the *rpm1*-independent response is not related to the *RPM1*-dependent response (Z. N. et al., unpublished).

Figure 4B demonstrates that both AvrRpm1 and AvrB accumulate in a DEX-dependent manner in Mt-0 and Fe-1 leaves. Figure 4C demonstrates that the *rpm1*-independent response to AvrB is abolished by G2A exchange. AvrB protein levels in plants expressing *rpm1*-independent phenotypes decline rapidly, preceding onset of the visible phenotype. However, Figure 4D demonstrates that the phenotypic difference between wild-type and G2A exchange AvrB proteins is not due to differential intrinsic stability, at least over a 48 hr induction time course in the nonresponding Cvi-0 accession. We conclude that AvrB can consistently initiate a slow, *rpm1*-independent response in some, but not all, *Arabidopsis* genetic backgrounds. G2A exchange greatly diminishes this response, like the *RPM1*-dependent responses described above. Because this *rpm1*-independent response is also greatly enhanced by consensus acylation sites, we conclude that it reflects both a normal function and cellular localization of this type III effector protein.

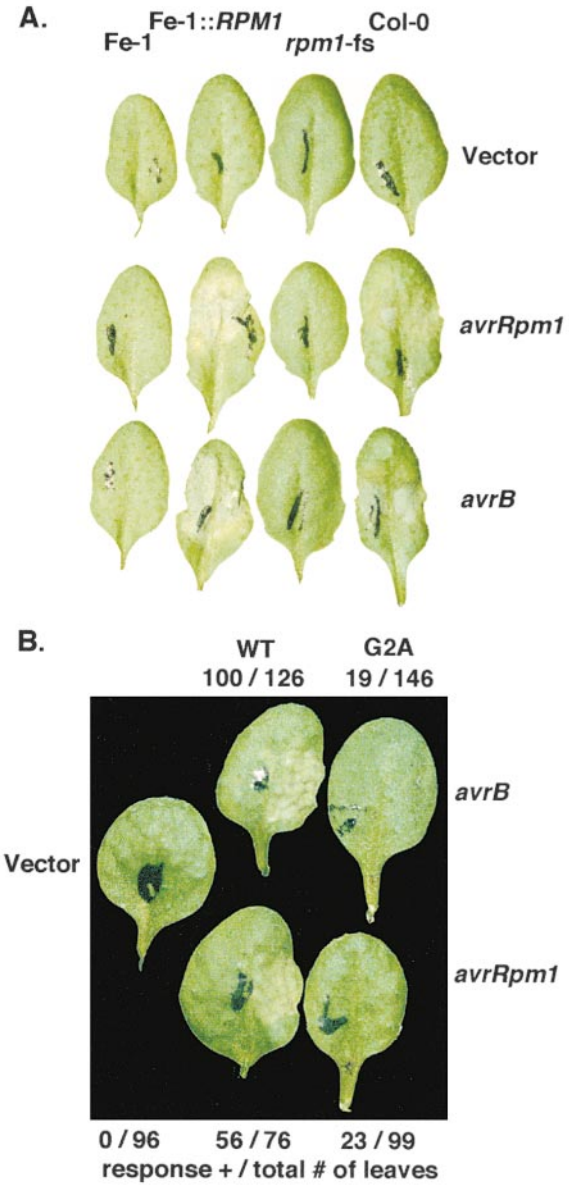

Figure 3. Myristoylation Sites Are Required for Maximal Avirulence Function inside Plant Cells

(A) AvrRpm1 and AvrB initiate RPM1 action inside plant cells. *Agrobacterium* carrying the genes listed at right were inoculated at OD_{600} = 0.5 and leaves sprayed with 20 μM DEX 48 hpi. *RPM1*-dependent responses were photographed 24 hr post DEX treatment. Plant accessions listed across top are: Fe-1 (*rpm1* null), Fe-1::*RPM1* (transgenic Fe-1 expressing *RPM1*), *rpm1*-fs (a Col-0 mutant *RPM1* allele), Col-0 (wild type, *RPM1*). (B) Consensus myristoylation sites greatly enhance AvrB and AvrRpm1 effector function inside the plant cell. Col-0 (*RPM1*) leaves were inoculated with *Agrobacterium* (OD_{600} = 0.4) carrying wild type (wt) or G2A mutant (top) of either *avrRpm1* or *avrB* (listed at right). Experiment done as in (A). Numbers above or below each leaf refer to *RPM1*-dependent response positive leaves/total number inoculated; data pooled from four experiments. In all cases, native *avr* gene constructs gave identical phenotypes compared to HA epitope–tagged constructs.

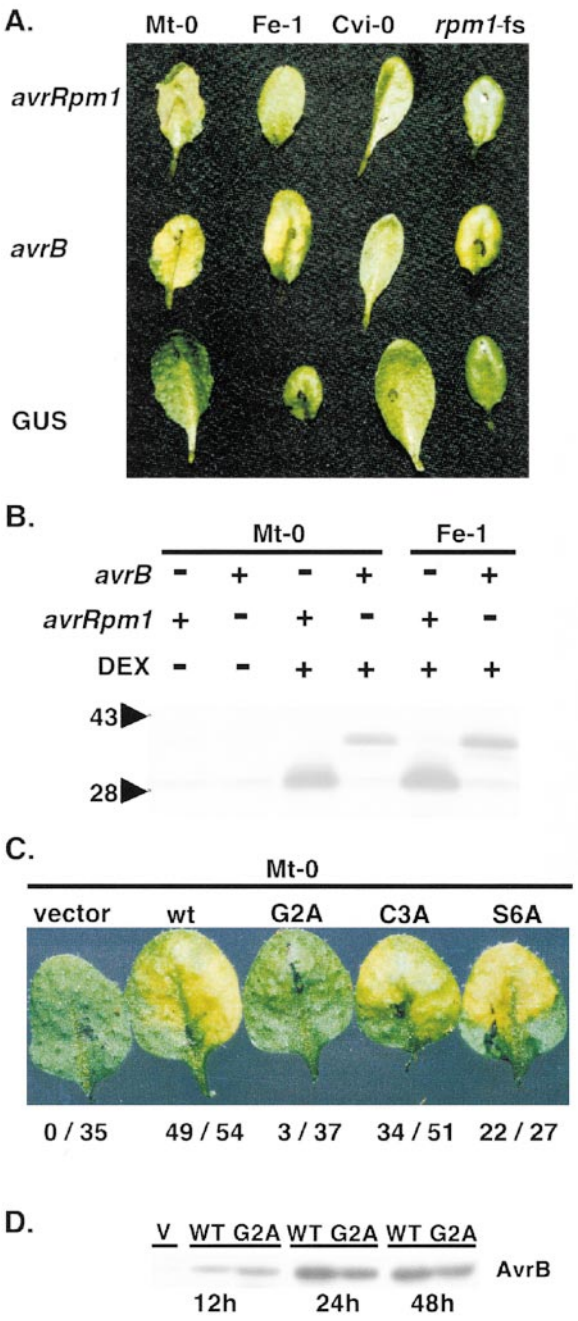

Figure 4. Expression of AvrRpm1 and AvrB in *rpm1* Plants

(A) Definition of an *RPM1*-independent, polymorphic response to AvrB expression in a series of *rpm1* null plants. *Agrobacterium* carrying the genes listed at left were inoculated into *rpm1* null accessions Mt-0, Fe-1, or Cvi-0, or the *rpm1*-fs mutant listed across the top. DEX induction as in Figure 3A, but photographed at 72 hr post DEX treatment. GUS expression in planta was utilized as a control for transformation. (B) DEX-dependent expression of AvrRpm1 and AvrB results in detectable protein accumulation in *rpm1* null plants. Protein extracts (10 μg) taken from two leaf discs from either *rpm1* null accessions Mt-0 or Fe-1, at 8 hr post DEX treatment, were subjected to SDS-PAGE and immunodetected using anti-HA monoclonal antibody. Molecular weight standards are marked at left. (C) Robust AvrB-induced host response requires G2. The *rpm1* null accession Mt-0 was inoculated with *Agrobacterium* carrying vector, wild type (wt) or mutant *avrB* derivatives as listed on the top. Numbers below the leaves represent response +/total inoculated pooled from three independent experiments. (D) Wild-type and G2A mutant AvrB proteins are equally stable. The *rpm1* null accession Cvi-0 was inoculated with *Agrobacterium* carrying vector, wild-type (WT) or the G2A mutant *avrB* derivative as listed on the top. Total protein extracts (10 μg) from two leaf discs were harvested at 12 hr, 24 hr, and 48 hr post DEX treatment, subjected to SDS-PAGE, and immunodetected using anti-HA monoclonal antibody. Similar results were seen in separate experiments.

AvrRpm1 and AvrB Are Localized to the Host Cell Plasma Membrane, and Efficient Localization Requires Consensus Acylation Sites

Our results suggested that Avr protein localization dictates both *RPM1*-dependent responses to both AvrRpm1 and AvrB, and the *rpm1*-independent response to AvrB. We localized both HA-tagged Avr proteins in Cvi-0 (Figure 5A) and Mt-0 (not shown) at 12 hr post DEX induction. We chose this time point because wild-type AvrRpm1 was undetectable at later time points in all tested accessions (not shown), and wild-type AvrB was undetectable in Mt-0 beyond this time point. We collected total extracts and prepared soluble and 100,000 × g microsomal fractions after DEX induction (Experimental Procedures). The anti-HA epitope monoclonal antibody detected bands of the correct apparent molecular weight (AvrRpm1 at 29 kDa, AvrB at 36 kDa) almost exclusively in the microsomal membrane fraction (antisera to AvrRpm1 and AvrB confirmed these results, not shown). These bands are not present in extracts from empty vector controls. G2A or C3A exchange had significant effects on membrane localization of both AvrRpm1 and AvrB. First, G2A exchange essentially eliminated membrane localization. Second, the C3A exchange significantly reduced membrane association. These results are precisely those expected given the requirement for myristoylation to occur before palmitoylation, but not vice versa, in various dually acylated proteins (see Discussion). These data also demonstrate that the localization differences observed are not due to differential stability of the mutant proteins, at least at 12 hr post DEX induction. Antisera against the tonoplast membrane protein γ-TIP served as a control for fractionation (Daniels et al., 1994).

We performed two-phase membrane vesicle separation to determine if the plasma membrane contains wild-type AvrRpm1 and AvrB (Experimental Procedures). Western blots (Figure 5B) demonstrate that both Avr proteins were enriched in these vesicles to the same extent as a known plasma membrane marker. This is consistent with previous enrichment of RPM1 protein in plasma membrane vesicles (Boyes et al., 1998). Marker proteins for various subcellular membranes confirmed the two-phase separation efficiency (Experimental Procedures). We used an independent method to confirm these results. Wild type and G2A exchange derivatives of either AvrRpm1 or AvrB were fused with green fluorescence protein (GFP) at their carboxyl termini and expressed from the strong cauliflower mosaic virus 35S promoter in *Arabidopsis rpm1* mutant protoplasts (see Experimental Procedures). Stacked laser confocal micrographs (Figure 5C) clearly demonstrate that the wild-type AvrRpm1 and AvrB proteins were enriched in the plasma membrane while their respective G2A derivatives localized like a nontargeted GFP control, mostly to the cytoplasm.

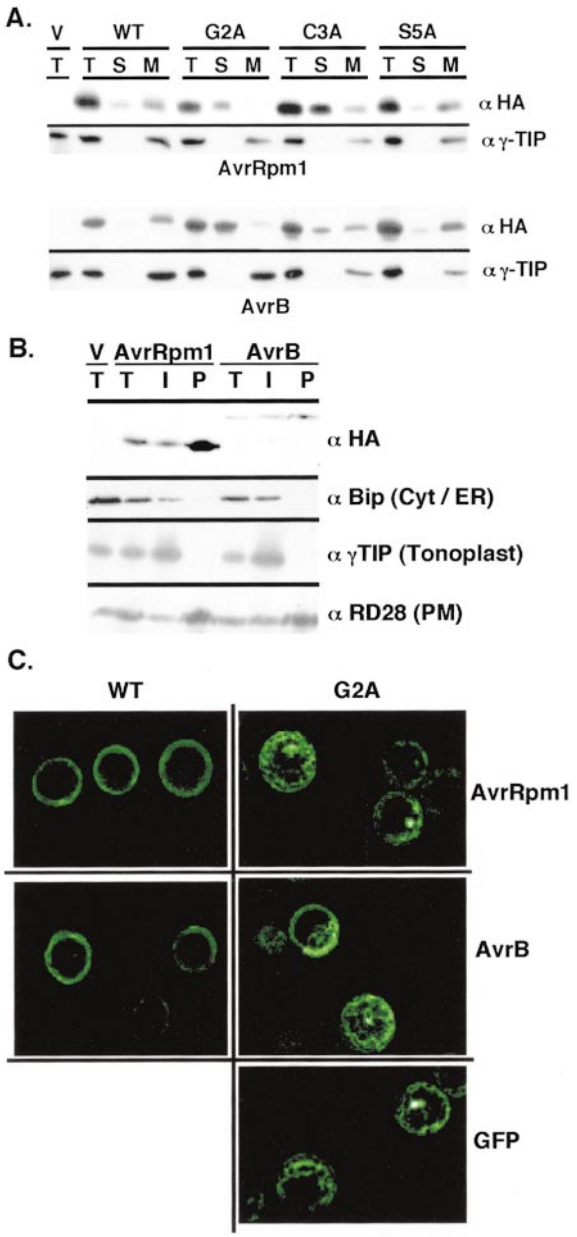

Figure 5. AvrRpm1 and AvrB Localize to a Plant Cell Membrane Fraction

(A) Localization is facilitated by consensus myristoylation and palmitoylation sites. The *rpm1* null accession Cvi-0 was inoculated with *Agrobacterium* carrying vector (V), wild type (WT) or the mutant *avr* derivatives listed across the top. DEX induction as in Figure 3A. Leaves were harvested 12 hr post DEX treatment. Total (T) extracts were separated into soluble (S) and 100,000 × g microsomal pellet (M) fractions, subjected to SDS-PAGE, blotted, and probed with anti-HA epitope monoclonal antibody to detect either AvrRpm1 (experiment in top set of two blots) or AvrB (bottom set of two blots), or with antisera against the tonoplast membrane marker γ-TIP (both sets of blots). All apparent molecular weights are correct (AvrRpm1 at 29 kDa, AvrB at 36 kDa, γ-TIP at 27 kDa). (B) AvrRpm1 and AvrB are highly enriched in plasma membrane vesicles. The *rpm1* null accession Cvi-0 was inoculated with *Agrobacterium* carrying vector (V) or wild-type *avr* genes listed across the top. Total (T) vesicles were separated by two-phase enrichment into intracellular (I) and plasma membrane enriched (P) pools. Equal yields were electrophoretically separated, blotted, and probed with the anti-HA monoclonal to detect either AvrRpm1 or AvrB, or with antisera against markers known to reside in the cellular compartments listed at right (Cyt is cytosol, ER is endoplasmic reticulum, PM is plasma membrane). All apparent molecular weights are correct (Bip at 70 kDa; γ-TIP at 27 kDa; RD28 at 27 kDa). (C) AvrRpm1 and AvrB green fluorescence fusion proteins localize to the protoplast plasma membrane. Protoplasts from *rpm1* plants were transformed with plasmids that express either wild type (WT) or G2A derivatives of AvrRpm1 or AvrB. Control transformations were with GFP alone. Stacked laser confocal micrographs are presented. The experiment was repeated many times with the same result (see Experimental Procedures).

Myristoylation of AvrRpm1 and AvrB In Vivo Requires Consensus G2 Acylation Sites

Our functional and plasma membrane localization data strongly support the contention that AvrRpm1 and AvrB are acylated, and thus tethered into the eukaryotic host plasma membrane. We tested myristoylation directly by radiolabeling leaves with [^{3}H]myristic acid subsequent to *Agrobacterium* inoculation and DEX induction. We prepared total protein extracts for fractionation and immunoprecipitation (Figure 6). The wild-type and G2A exchange mutants for either Avr protein were equally represented in the extracts, yet ^{3}H was only incorporated into the wild-type AvrRpm1 and AvrB proteins. Thus, these proteins can be myristoylated in vivo. Coupled with the functional role for a G2 residue for complete expression of all tested functions and for efficient plasma membrane localization of both AvrRpm1 and AvrB, it is likely that myristoylation is essential for both optimal function and localization of AvrRpm1 and AvrB.

Proteolytic Processing of a *P. syringae* Type III Effector in Host Cells Exposes a Eukaryotic N-Myristoylation Consensus Site

The 35 kDa AvrPphB protein from *P. syringae* pv. *phaseolicola* is rapidly cleaved between K62 and G63, in both *E. coli* and *P. syringae* (Puri et al., 1997). The longer, 28 kDa product of this cleavage exposes a potentially myristoylated free glycine at its N terminus (Table 1). To generalize our findings with AvrRpm1 and AvrB, we constructed a G63A *avrPphB* mutation for expression of either native or HA-tagged derivatives in the DEX-inducible *Agrobacterium* system. The 28 kDa cleavage product consistently accumulates following DEX-induced transient expression of AvrPphB in leaves. We have occasionally observed very low levels of the 35 kDa translation product in soluble fractions (not shown), suggesting that it is also rapidly processed in plant cells. The 28 kDa cleavage product localizes to a membrane fraction in a G63-dependent manner (Figure 7). This residue also greatly enhances recognition of AvrPphB by *RPS5* (Warren et al., 1998) following *Agrobacterium* delivery (responding leaves: 59/70 for wild type and 13/74 for G63A), consistent with a functional role for myristoylation in membrane localization of AvrPphB in the plant cell. We conclude that AvrPphB can be cleaved in the plant cytoplasm to an active 28 kDa form, which utilizes a myristoylation site for both localization to a membrane compartment and recognition by *RPS5*.

Discussion

Bacterial pathogens of both plants and animals use type III secretion systems to deploy effector proteins into

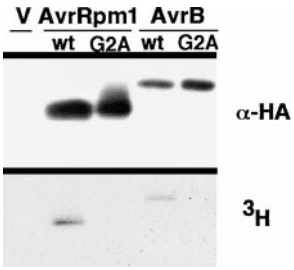

Figure 6. AvrRpm1 and AvrB Are Myristoylated In Vivo in a G2-Dependent Manner

The *rpm1* null accession Cvi-0 was inoculated with *Agrobacterium* carrying vector (V), wild type (WT), or G2A derivatives of *avrRpm1* or *avrB* listed at the top. At 48 hpi, an ~5 μM [^{3}H]myristic acid/20 μM dexamethasone solution was hand inoculated into the *Agrobacterium*-infiltrated leaves. Total extracts were prepared 12 hr later. Total extract from two leaf discs was immunoprecipitated with anti-HA monoclonal. Equal yield aliquots were either immunoblotted as in Figure 5, or analyzed by fluorographically enhanced autoradiography for 3 weeks.

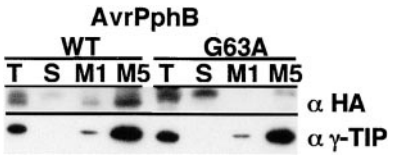

Figure 7. An Embedded Consensus Myristoylation Site at G63 Mediates AvrPphB Membrane Localization

Accession La-*er* (*rps5*) leaves were inoculated with *Agrobacterium* carrying empty vector (V), wild type (WT) or a G63A exchange derivative of *avrPphB*. DEX induction as in Figure 3A. Samples were harvested 6 hr post induction and processed as in Figure 5A. M5 refers to 5× more than the equal yield amounts loaded in the other lanes.

eukaryotic host cells. These effector proteins are positioned to interact with and regulate specific components of host signaling networks, and are targets for modification by host cellular components. Avr proteins are type III–dependent effectors of disease and triggers of plant disease resistance. We identified eukaryotic N-terminal myristoylation and palmitoylation consensus sequences on AvrRpm1, AvrB, AvrC, and AvrPto, and we noted that posttranslational cleavage exposes a eukaryotic consensus acylation site on AvrPphB. We demonstrated that consensus myristoylation sites are required for maximal function of AvrRpm1 and AvrB when delivered from *P. syringae* to *Arabidopsis*. We also demonstrated that both the consensus myristoylation and palmitoylation sites of AvrRpm1 are required for maximal virulence of the *P. syringae* strain Psm CR299. Additionally, the myristoylation sites of AvrRpm1, AvrB, and AvrPphB enhance *R* gene–specific responses when each Avr protein is expressed inside host cells. Thus, the genetically defined role for these consensus acylation sites is independent of the type III secretion machinery.

Function correlates with membrane localization and myristoylation of the Avr proteins, and the major membrane system targeted for AvrRpm1 and AvrB is the plasma membrane. We documented a host genotype–specific, *rpm1*-independent response to AvrB expression. Importantly, this response is also greatly enhanced by membrane association mediated by the consensus myristoylation site. We propose that this slow cytotoxic response could reflect the AvrB function in promoting disease on *rpm1* plants. We were unable to detect either AvrB or AvrRpm1 protein in host cells at the time when *rpm1*-independent responses were observed. However, there is no intrinsic difference in the stability of wild-type and mutant AvrRpm1 or AvrB proteins at either a time point preceding the onset of this response, or for AvrB in a nonresponding plant at time points beyond the onset of the *rpm1*-independent response. Thus, the differential phenotypes of wild-type and mutant Avr proteins are probably not due to differential intrinsic stability of each protein in host cells.

We provide compelling in vivo evidence that AvrRpm1 and AvrB are myristoylated in a G2-dependent manner. Protein palmitoylation is widely believed to be post-translational and palmitoyltransferase activity is enriched in plasma membranes (Dunphy et al., 1996). Palmitoylation often requires previous myristoylation (Berthiaume and Resh, 1995). Myristoylation is viewed as a cotranslational process: cyclohexamide treatment abolishes myristoylation (Olson and Spizz, 1986); nascent polypeptide chains associated with tRNAs have myristate covalently attached (Wilcox et al., 1987); and a significant fraction of N-myristoyltransferase is associated with ribosomes (Glover et al., 1997). Pathogen effector proteins delivered into host cells via the type III apparatus would thus appear to be unsuitable substrates for myristoylation. Yet, the necessity of cotranslational myristoylation was initially challenged by incorporation of radiolabelled myristate when protein synthesis is inhibited (da Silva and Klein, 1990) and by the finding of N-myristoyltransferases in both the endoplasmic reticulum and the cytosol (Boutin, 1997).

The proteolytic cleavage product of AvrPphB associates with a membrane fraction in a G63-dependent manner following processing from the full-length wild-type protein. Pulse–chase studies in *P. syringae* revealed that full-length AvrPphB is rapidly processed (Puri et al., 1997) and our data suggest that the same is true in plant cells. Myristoylation of the exposed N-terminal glycine of the larger AvrPphB cleavage product would thus follow synthesis of full-length protein, and subsequent cleavage exposing G63. These constraints make cotranslational acylation of AvrPphB unlikely and support the notion that some proteins are capable of posttranslational myristoylation. We cannot, however, exclude cotranslational myristoylation of AvrB and AvrRpm1. Exposure of G2 by removal of the initiator methionine for AvrRpm1 and AvrB could be achieved by methionine aminopeptidases inside either the host cell or the bacteria prior to delivery, as reported for the type III effector proteins TIR and SopE (Wood et al., 1996; Kenny et al., 1997).

AvrPto (Table 1) can localize to a plasma membrane fraction (T. L. and F. K., unpublished; X. Tang personal communication). Pto, which recognizes AvrPto, contains a G2 residue. However, site-directed G2A exchange did not alter Pto function when overexpressed (Loh et al., 1998). Wild-type Pto expressed from its own promoter has not been localized. Yet, the Pto-related Fen kinase has a demonstrated G2 requirement for function, and a chimeric Fen possessing a Pto N terminus

still functions. Thus, the N terminus of Pto is capable of providing a myristoylation site required for Fen function (Rommens et al., 1995). Cytoplasmic Pto may sequester incoming AvrPto, before the latter is localized. Alternatively, overexpressed, nonmyristoylated Pto may be recruited to the membrane by AvrPto as observed for $G\alpha_z$/$B\gamma$ subunit interactions (Morales et al., 1998). This would be consistent with our unpublished observations that constitutive overexpression of the G2A Avr derivatives eliminated phenotypic differences with wild type.

Subcellular localization of *P. syringae* type III effector proteins in a host cell probably facilitates virulence. The decreased functions of G2A and C3A mutants of AvrRpm1, for Psm M2 virulence, and of AvrB, for the *rpm1*-independent response, support this view. Plasma membrane localization may mirror that of the host targets of AvrRpm1 and AvrB disease effector function. Localization of RPM1 to the *Arabidopsis* plasma membrane is consistent with either direct or indirect recognition of membrane-associated AvrRpm1 or AvrB. Other *P. syringae* Avr proteins lack acylation sequences, suggesting variable subcellular sites of action for incoming type III effectors. This variation could favor evolution of host recognition complexes, anchored by the relevant *R* product, with different subcellular localizations. If this model is correct, it predicts that a major function of *R* products is to "guard" subcellular targets of type III effector proteins. This model, first elaborated by Van der Biezen and Jones (1998) further predicts that the cellular targets of effector action during disease onset would also be found in an R protein complex in resistant host genotypes. By contrast, if each structural class of R protein assembled into a defense-dedicated protein complex, then common elements should be found in the signalasome of various R proteins. Thus far, yeast two-hybrid analyses with various NBS-LRR class R proteins have failed to identify such a uniform set of proteins.

Interestingly, while the putative palmitoylation site at C3 is not absolutely required for AvrRpm1 triggering of RPM1 function, it is required for the virulence function of AvrRpm1 on *rpm1* null hosts. Mutation at C3 partially reduces, but does not abolish, AvrRpm1 membrane localization, while the G2A exchange has a severe localization defect. This is consistent with studies demonstrating that myristoylation defects prevent subsequent palmitoylation, but that the reverse is not true (McCabe and Berthiaume, 1999). RPM1 function is probably sensitive to very small quantities of Avr protein at the membrane, congruent with recent evidence that increasing the level of Avr or R protein can increase the sensitivity of the overall disease resistance response (Bendahmane et al., 1999). In contrast, the virulence function of AvrRpm1 might operate via a more stable association with the membrane, achieved through additional palmitoylation, or quantitatively by delivery of more protein to the membrane. Alternatively, the putative palmitoylation may serve a regulatory role as demonstrated for the modulation of GAP activity by palmitoylated $G\alpha$ subunits (Tu et al., 1997). In this scenario, palmitoylation of AvrRpm1 C3 could differentially affect *RPM1*-dependent and *rpm1*-independent functions.

The host targets of type III effectors of phytopathogen virulence, and the means by which they modulate or usurp host cell signal pathways to promote disease, are largely unknown. That acylation and membrane localization are not general features of type III effectors is illustrated by our finding that, of 46 putative or known type III effectors from animal and plant pathogens (Hueck, 1998), none carry consensus acylation sequences at their N termini. Of course, some of these, like AvrPphB, could be proteolytically processed to reveal an acylation site. We demonstrated that expression of AvrB induces cytotoxicity in some, but not all, *rpm1* null plants. These cytotoxic effects may be indicative of the virulence function of AvrB in host cells. Note that the inability to ascribe a virulence function to AvrB in the context of Pst DC3000–induced disease does not alter this conclusion, as it could be the case that AvrB is redundant to an unknown type III effector in DC3000. The polymorphic nature of the host response, and our ability to map a locus responsible for it, indicates that this is a specific effect. Several other Avr type III effectors can induce cytotoxic effects on disease-susceptible host cells using similar expression-based systems. In animal systems, expression of various type III effector molecules in host cells is known to phenocopy aspects of the host disease response and in many cases this effect can be traced to interactions with relevant host cell targets (Hueck, 1998). Our ability to isolate *Arabidopsis* mutants in the *rpm1* null Mt-0 background that fail to respond to AvrB expression (Z. N. and J. L. D., unpublished) should enrich our understanding of the role these proteins play in inducing disease and altering host cell physiology.

Experimental Procedures

Construction of *avr* Clones and Mutants

Site-directed mutants (Chameleon System) were sequenced for verification. DEX-inducible HA-tagged wild-type and mutant *avr* genes were cloned into pTA7002 (Aoyama and Chua, 1997) for expression in planta. For expression in *P. syringae*, *avrB* and *avrRpm1* constructs are cloned behind the native *avrRpm1* promoter (Ritter and Dangl, 1995) in pVSP61 (Bisgrove et al., 1994). *avrB* and *avrRpm1* were cloned into pDSK519 for expression in *E. coli*. Details available upon request.

P. syringae HR and In Planta Growth Assays

For in planta inoculations (Debener et al., 1991), *P. syringae* were resuspended to $OD_{600} = 0.1$ (corresponding to $\sim 5 \times 10^7$ cfu/ml) for HR assays or diluted to 1×10^5 cfu/ml for growth curves. HR was scored from 5 hr for *avrRpm1* and *avrB*.

P. syringae Protein Extraction and *E. coli* Secretion Assays

2.5 ml overnight Pst DC3000 cultures in KB with the appropriate antibiotics were pelleted, washed with *hrp* gene–inducing media (Ritter and Dangl, 1995), resuspended in 2.5 ml of the same, and induced for 5 hr. Cultures were spun down and resuspended in 500 μl 65°C, 3× Laemmli buffer. Samples were boiled 2 min and 5 μl loaded on an SDS-PAGE.

Agrobacterium Transient Expression Assays

2 ml overnight *Agrobacterium* cultures were grown at 30°C in YEB (5 g bacto beef extract, 1 g bacto yeast extract, 5 g bacto peptone, 5 g sucrose, 2 mM $MgSO_4$, pH 7.2, per liter) containing 100 μg/ml each of rifampicin, kanamycin, and gentamycin for strain GV3101. The following day, 150 μl of saturated culture was inoculated into 3 ml of YEB plus antibiotics, and grown for 13 hr. Two milliliters was collected and resuspended in 3 ml *Agrobacterium* induction medium (10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g (NaCitrate), 1 mM $MgSO_4$, 1 g glucose, 1 g fructose, 4 ml glycerol, 10 mM MES, pH

5.6, per liter, 50 μg/ml acetosyringone), grown at 23°C for 5–7 hr, collected and resuspended in infiltration medium (1/2 MS-MES) to an OD_{600} of 0.4. The underside of 3-week-old leaves were inoculated using a needleless syringe. Plants were grown in >120 μE of light and sprayed with 20 μM DEX (Sigma, St. Louis, MO) 48 hr after inoculation, except for in planta myristoylation assays (see below). *RPM1*-dependent or *RPS5*-dependent responses were scored 24 hr later, and the *rpm1*-independent responses scored 2–3 days later. All phenotypes noted were confirmed to be dependent on T-DNA transfer by testing constructs in a GV3101 strain cured of the *vir* plasmid (data not shown).

Plant Protein Extractions

Two 6 mm diameter leaf discs were ground in a 1.5 ml Eppendorf tube in 50 μl protein extraction buffer (20 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1× plant protease inhibitor cocktail (PIC; Sigma, St. Louis, MO), and 10 μl 6× Laemmli buffer (final concentration 1×) was added. Samples were vortexed, boiled for 3 min, then spun briefly. Ten microliters was loaded on an SDS-PAGE gel.

Total Plant Membrane Fractionation

Fifteen 6 mm leaf discs per sample were ground in 200 μl membrane extraction buffer (MEB) (10 mM Tris-HCl pH 7, 0.33 M sucrose, 1 mM EDTA, 1× PIC) in a 1.5 ml Eppendorf tube. Three hundred microliters of MEB was added, and samples vortexed and cleared at 8,000 × g for 3–4 min. Four hundred fifty microliters of supernatant was transferred to an Eppendorf tube containing 10 μl 1 M $CaCl_2$. A 50 μl aliquot was removed as the "total extract" fraction and the remainder was spun 50,000 × g for 1.5 hr. The resulting supernatant (soluble fraction) was prepared for SDS-PAGE. The pellet (membrane fraction) was resuspended in 460 μl TE with PIC. For each fraction, 10 μl of 6× Laemmli buffer was added to a 50 μl aliquot and 10 μl of this sample was loaded to obtain equal yield. Proteins were separated using SDS-PAGE on a 15% polyacrylamide gel.

Plasma Membrane Purification

Plasma membrane and intracellular vesicles were prepared using aqueous two-phase partitioning as described in Boyes et al. (1998). Equal protein levels from each fraction were loaded on the gel. Antibodies used for membrane localization were raised against RD28 (plasma membrane), γ-TIP (tonoplast), (gifts of Maarten Chrispeels), BiP (intercellular membranes and cytosol; gift of Rebecca Boston), and an antibody to the HA epitope (Roche Biochemicals, Indianapolis, IN). All antibodies were used at a dilution of 1:1000.

In Planta Myristoylation Assay

Agrobacterium cultures were grown and induced as described above. *Avr* induction and labeling of total proteins with [^{3}H]myristate was performed by coinoculating a solution of 20 μM dexamethasone and 500 μCi/ml [9,10–^{3}H(N)] myristic acid (American Radio Chemicals, St. Louis, MO) directly into the underside of leaves using a needleless syringe 48 hr after *Agrobacterium* inoculation. After 12 hr, two leaf discs per sample were ground in IP buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM $NaCl_2$, 1% Triton X-100 [v/v]), and 1% plant PIC. Crude lysate was spun down (2 min, 4°C, 10,000 × g) and the supernatant was immunoprecipitated using 30 μl of High Affinity HA-Antibody coupled matrix (Roche Diagnostics, Indianapolis, IN) and end-over-end tumbling on a rotary motor for 2 hr at 4°C. Matrix was pelleted by spinning 2 min, 4°C, 10,000 × g. Matrix was then washed and pelleted twice with 500 μl ice-cold IP solution and finally resuspended in 50 μl SDS-protein extraction buffer (see above) and 10 μl of 6× gel loading buffer. Ten-microliter aliquots were loaded onto gels and separated as described above. Proteins were identified on Western blots with mouse, anti-HA antibody and subsequent detection with mouse-secondary conjugated to peroxidase as described above. For radiolabel detection, gels were subjected to fluorographic impregnation using Amplify (Amersham International), dried down, and exposed to X-ray film.

Plasmid Construction for GFP Microscopy

Transient expression constructs are in pKEx4tr (Leister et al., 1996). C-terminal green fluorescent protein (GFP) fusions were obtained by fusing the synthetic (GFP) coding sequence to the coding sequences in pEx*avrRpm1* and pEx*avrB*. pExGFP was constructed by cloning the GFP coding sequence into pKEx4tr. Site-directed mutagenesis of the G2A residues in *avrRpm1* and *avrB* used the respective wild-type *avr*-GFP fusions and specific primers as template. Details of the cloning procedures are available upon request.

Protoplast Preparation and Transformation

Arabidopsis plants from a cross between ecotype Niederzenz (Nd-0) (*rpm1*/*rpm1*) and rps2–101C (*rps2–101*/*rps2–101C*; Col-0 background), were used for all protoplast studies as in Leister et al. (1996). *Arabidopsis* leaf mesophyll protoplasts were prepared from 5-week-old plants and transformed using polyethylene glycol. Transformation efficiency (i.e., percentage of GFP-positive protoplasts) was 30%–40% with pExGFP. Protoplasts were transformed with either 3.0 μg pExGFP, 3.0 μg p*avrB*G2A-GFP, 6.0 μg p*avrB*-GFP, 6.0 μg p*avrRpm1*-GFP, or 6.0 μg p*avrRpm1*G2A-GGFP. pKEx4tr-dGFP (a defective GFP; Leister et al., 1996) was added where needed to keep the total amount of DNA equal for each treatment. Following transformation, the protoplasts were incubated overnight in the light at room temperature.

Microscopy

Protoplasts were examined on a Leica model DMIRBE confocal microscopy system appropriate for the detection of S65T GFP using an argon laser for excitation (488 nm) and the BP-FITC detector setting for collection. Images were generated using the extended focus option under 3D-image processing. Each image represents the scaled summation of ten optical sections beginning 5 μm from the bottom of the cell and continuing toward the center of the cell. Each optical section is approximately 0.5 μm in thickness.

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